Telomere length is reset during early mammalian embryogenesis

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The enzyme telomerase is active in germ cells and early embryonic development and is crucial for the maintenance of telomere length. Whereas the different length of telomeres in germ cells and somatic cells is well documented, information on telomere length regulation during embryogenesis is lacking. In this study, we demonstrate a telomere elongation program at the transition from morula to blastocyst in mice and cattle that establishes a specific telomere length set point during embryogenesis. We show that this process restores telomeres in cloned embryos derived from fibroblasts, regardless of the telomere length of donor nuclei, and that telomere elongation at this stage of embryogenesis is telomerase-dependent because it is abrogated in telomerase-deficient mice. These data demonstrate that early mammalian embryos have a telomerase-dependent genetic program that elongates telomeres to a defined length, possibly required to ensure sufficient telomere reserves for species integrity.

The enzyme telomerase is active in germ cells and during early embryogenesis (1–5) and is crucial for the maintenance of telomere length and germ cell viability in successive generations of a species (6, 7). Telomere shortening limits the regenerative capacity of cells and is correlated with the onset of cancer, aging, and chronic diseases (8–12). The segregation of telomere length from one generation to the next seems to be essential for normal development and well being in mammals. In line with this hypothesis, telomere shortening in telomerase knockout ($mTERC^{-/-}$) mice is correlated with a variety of phenotypes including impaired organ regeneration, chromosomal instability, and cancer (9, 13–16).

Telomere length segregation is ensured by the active elongation and maintenance program in germ cells. Mature germ cells possess significantly longer telomeres compared to somatic cells (17), possibly because of telomere elongation during germ cell maturation (18). In contrast to telomere elongation in germ cells, little is known about telomere length regulation during embryogenesis. Establishment of telomere length during embryogenesis could determine telomere reserves in newborns. In newborn mice derived from crossbreeding of late generation $mTERC^{-/-}$ mice and $mTERC^{+/-}$ mice, telomere function is rescued correlating with the elongation of critically short telomeres (19). Similarly, mice derived from intercrosses of mouse strains with different telomere lengths show a telomerase-dependent elongation of those telomeres derived from the strain with shorter telomeres (20, 21). These observations can be explained only if the telomeres are elongated during embryogenesis. In line with this hypothesis, studies on cloned mice and cattle have revealed telomere length restoration of cloned animals even when senescent fibroblasts had been used as the donor cells (22–25). In contrast, cloned sheep derived from epithelial cells had shorter telomeres than control animals (26). Telomere length rescue in cloned animals seems to depend on the used donor cell type (27). Alternatively, rather than telomere restoration during embryogenesis, cloned animals may be derived from the selective propagation of cells with enhanced telomere reserves during the cloning process and/or early embryonic development, which would be consistent with the low efficiency of somatic cloning. If telomere restoration in fact occurs during preimplantation development, it remains to be investigated whether this restoration is related to the cloning process itself or represents a general mechanism during embryogenesis to ensure the presence of adequate telomere reserves for species integrity. To test whether telomere length is regulated during embryogenesis, we have analyzed telomere length at different stages of bovine and mouse early development employing embryos derived from somatic nuclear transfer, *in vitro* production (*in vitro* maturation, fertilization, and culture), and conventional breeding. Results show that telomere length is determined at morula to blastocyst transition by a telomerase-dependent mechanism. Telomere elongation is restricted to this stage of development and restores telomeres of fibroblast-derived cloned embryos to normal length.

Methods

Quantitative Fluorescence *in Situ* Hybridization (Q-FISH) on Interphase Nuclei. Q-FISH was performed on interphase nuclei as described on nuclei of adult and fetal fibroblasts (11, 28). Nuclei were isolated by cell lysis with 0.075 M KCl and fixed in glacial methanol/acetic acid (3:1). Bovine adult and fetal fibroblasts and a human transformed kidney cell line (Phoenix cells, a gift from Gary Nolan, Stanford University, Stanford, CA) were analyzed in parallel with Q-FISH and Southern blotting to validate Q-FISH data. Results correlated closely between the two methods. To ensure linearity of the Q-FISH method, telomere length of liver cells from different generations of telomerase-deficient mice were analyzed by Q-FISH showing the expected decrease in telomere length from one generation to the other (data not shown).

Telomere Restriction Fragment (TRF) Length Analysis. TRF were determined in skin biopsies and blood samples (WBC) of cloned and conventionally produced cattle as well as bovine *in vitro* matured oocytes (see below) and bovine semen as controls. TRF length analysis was done as described in ref. 28. The mean TRF length was calculated by measuring the signal intensity in 10 squares covering the entire TRF smear. All calculations were performed with PCBAS and EXCEL (Microsoft) computer programs.

Telomeric Repeat Amplification Protocol. To measure telomerase activity in early embryos, a telomeric repeat amplification protocol assay was performed with a TRAPeze telomerase detection kit (Intergen, Purchase, NY) according to recommendations of the manufacturer.

Abbreviations: Q-FISH, quantitative fluorescence *in situ* hybridization; TRF, telomere restriction fragment.

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***In Vitro* Production of Bovine Embryos.** Bovine embryos were produced as described in refs. 29 and 30. Briefly, viable cumulus–oocyte complexes derived from abattoir ovaries were matured *in vitro* in groups of 15–20 in 100 μl of TCM-199 supplemented with 10 IU of pregnant mare serum gonadotropin and 5 IU of human chorionic gonadotropin (Suigonan, Intervet, Tönisvorst, Germany) and 0.1% BSA (Sigma, A7030) under silicone oil in a humidified atmosphere composed of 5% CO_2 in air at 39°C for 24 h. Matured cumulus–oocyte complexes were fertilized *in vitro* employing 1×10^6 sperm per ml of frozen/thawed semen from one bull with proven fertility in *in vitro* fertilization (29) during a 19-h coincubation under the same temperature and gas conditions as described for *in vitro* maturation. Presumptive zygotes were cultured up to the morula stage (day 6 after insemination) or blastocyst stage (day 8 after insemination) in synthetic oviduct fluid medium supplemented with BSA, in a mixture of 5% O_2, 90% N_2, and 5% CO_2 (Air Products, Hattingen, Germany) in modular incubator chambers (ICN).

***In Vivo* Production of Bovine Embryos.** *In vivo* grown bovine embryos were collected from superovulated donor animals inseminated with semen from the same bull as used for *in vitro* fertilization. Morulae and blastocysts were nonsurgically recovered from the genital tract of donors employing established protocols at days 6 and 8 after artificial insemination, respectively (31).

Generation of Nuclear Transfer-Derived Embryos. Bovine fetal fibroblasts were obtained from a 61-day female bovine fetus after evisceration and decapitation. Adult fibroblasts were established from ear skin biopsies of an adult female animal collected from a local abattoir. The fibroblasts used for nuclear transfer in these experiments were from passages 2–4 and were induced to enter a period of quiescence (presumptive G_0) by serum starvation for 2–3 days (0.5% FCS). Donor cells and cloned animals showed an identical microsatellite pattern (not shown). For enucleation and nuclear transfer, cytochalasin B (7.5 μg/ml) was added to TCM-air. For fusion, 0.285 M mannitol containing 0.1 mM $MgSO_4$ and 0.05% BSA was used. Embryos were reconstructed, and cloned offspring were produced as described in ref. 32.[¶] Briefly, *in vitro* matured oocytes were enucleated by aspirating the first polar body and the metaphase II plate. The donor cells were pelleted and resuspended in TCM-air and remained in this medium until insertion. A single cell was sucked into a 30-μm (outer diameter) pipette and was then carefully transferred into the perivitelline space of the recipient oocyte. Cell fusion was induced with 1–2 DC pulses of 0.7 kV/cm for 30 μs each generated by a Kruess electrofusion machine (CFA 400, Hamburg, Germany). At 27 h after onset of maturation, the reconstructed embryos were chemically activated by incubation in 5 μM ionomycin (Sigma) in TCM-199 for 5 min followed by a 3–4-h incubation in 2 mM 6-dimethylaminopurine (Sigma) in TCM-199 at 37°C. After three washes, the embryos were cultured for 6 (morulae) or 8 (blastocysts) days as described above. Morulae and blastocysts were used in Q-FISH for determination of telomere length.

Production of Mouse Embryos. Wild-type and telomerase knockout ($mTERC^{-/-}$, generation 2) females were killed on days 3 (eight-cell and morula stage) or 4 (blastocyst stage) of pregnancy. Day 0.5 is the morning after insemination. The embryos were collected from the oviducts or the uterus as described (33).

Statistical Programs. Student's *t* test and GRAPHPAD INSTAT software were used to calculate statistical significances and standard deviations.

[¶]Lucas-Hahn, A., Lemme, E., Hadeler, K. G., Sander, H. G. & Niemann, H. (2002) *Theriogenology* **57,** 433 (abstr.).

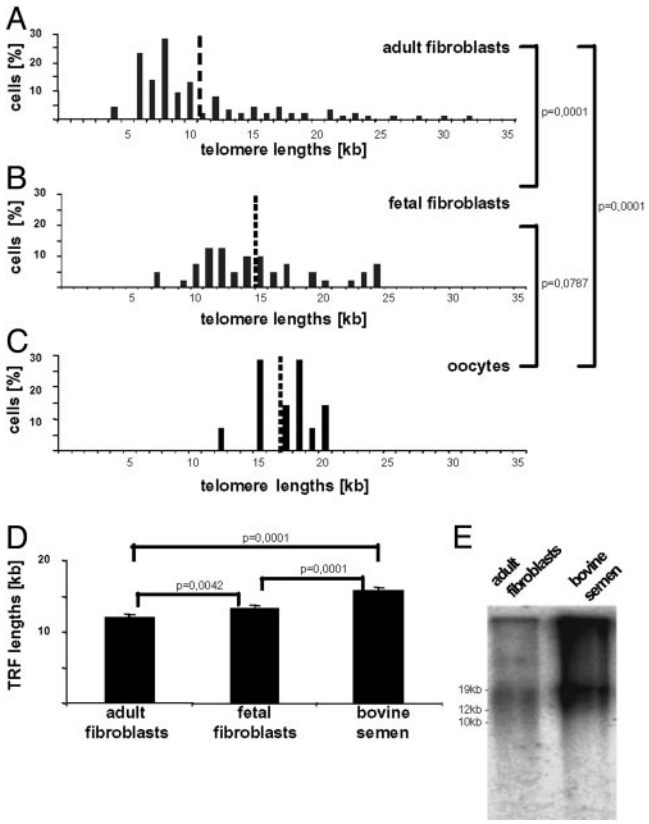

Fig. 1. Telomere length analysis of bovine germ cells and primary fibroblasts used for cloning. (*A*) Distribution of the mean telomere length as determined by Q-FISH in different nuclei of adult fibroblasts (total mean, 10.84 ± 5.73 kb; $n = 118$), (*B*) fetal fibroblasts (total mean, 14.61 ± 4.58 kb; $n = 40$), and (*C*) oocytes (total mean, 16.95 ± 2.51 kb; $n = 13$). (*D* and *E*) The length of TRFs was determined by Southern blotting on DNA of bovine fetal fibroblasts, adult fibroblasts, and semen. (*D*) Histogram on the mean value of TRF length as determined in triplicate. Telomere length was significantly shorter in both fibroblast cell lines (adult fibroblasts, 12.03 ± 0.46 kb; fetal fibroblasts, 13.37 ± 0.46 kb) used for cloning compared to bovine semen (15.83 ± 0.41 kb) used for *in vivo* or *in vitro* fertilization. (*E*) Representative radiograph of a Southern blot showing the TRF smear of adult bovine fibroblasts and semen. Dotted line in *A–C* indicates mean value.

All experiments were conducted according to the German animal welfare law and comply with international regulations.

Results

Telomere Length in Bovine Preimplantation Embryos at the Morula and Blastocyst Stage. We analyzed telomere length regulation during early embryogenesis in bovine embryos and animals derived from cloning or conventional production to elucidate whether the embryo has a defined telomere-elongation program. Q-FISH (Fig. 1 *A–C*) and Southern blot (Fig. 1*E*) analysis revealed significantly shorter telomeres in both adult and fetal fibroblasts that had been used for somatic nuclear transfer compared to bovine oocytes and semen serving as controls (Fig. 1 *C* and *E*). The telomere length of the adult fibroblasts was shorter than that of fetal fibroblasts (Fig. 1 *A*, *B*, and *D*). Telomere length was analyzed in morula stages, which are known to be telomerase negative or to possess minimum telomerase activity (3–5). Q-FISH analysis revealed significantly shorter mean telomeres in cloned morulae compared to their counterparts derived from *in vivo* or *in vitro* fertilization (Fig. 2). The finding of similar telomere lengths in morulae derived from *in vivo* and *in vitro* fertilization ruled out the possibility that the *in vitro* culture *per se* had a significant effect on telomere length (Fig. 2 *A*,

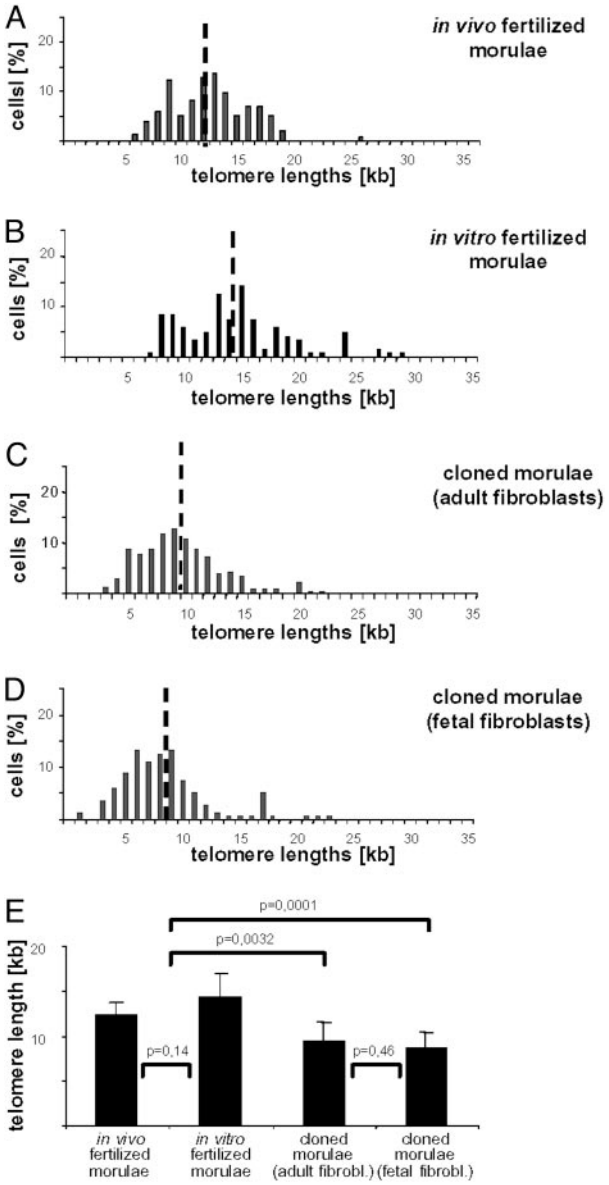

Fig. 2. Telomere length of cloned and fertilized bovine morulae by Q-FISH. (*A–D*). Distribution of mean telomere length over all analyzed nuclei of morulae (day 6 of embryonic development) derived from *in vivo* fertilization ($n = 234$) (*A*), *in vitro* fertilization ($n = 118$) (*B*), cloning from adult bovine fibroblasts ($n = 203$) (*C*), and cloning from fetal bovine fibroblasts ($n = 134$) (*D*). (*E*) Mean value of the mean telomere lengths determined for individual morulae derived by cloning from fetal fibroblasts (8.71 ± 1.81 kb, $n = 8$) or adult fibroblasts (9.47 ± 2.07 kb, $n = 6$) from *in vivo* fertilization (12.42 ± 1.37 kb, $n = 7$) or *in vitro* fertilization (14.36 ± 2.67 kb, $n = 4$). The difference in telomere length between morulae derived from *in vivo* and *in vitro* fertilization was not significant. Dotted line in *A–D* indicates mean value.

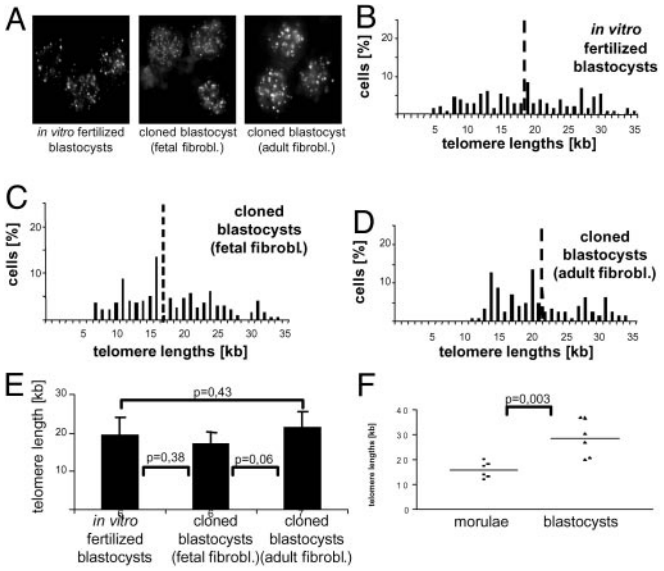

Fig. 3. Telomere length of cloned and fertilized bovine blastocysts by Q-FISH. (*A*) Representative photographs of telomere spots in nuclei of blastocysts (day 8 of development) derived from cloning of adult and fetal fibroblasts and *in vitro* fertilization. Magnification bar, 20 μm. (*B–D*) Distribution of mean telomere length over all analyzed nuclei of blastocysts derived from *in vitro* fertilization ($n = 129$) (*B*), cloning from fetal bovine fibroblasts ($n = 192$) (*C*), and cloning from adult bovine fibroblasts ($n = 126$) (*D*). (*E*) Mean value of the mean telomere lengths determined for individual blastocysts derived from *in vitro* fertilization (19.53 ± 4.6 kb, $n = 6$) and from cloning using fetal fibroblasts (17.3 ± 2.66 kb, $n = 6$) and adult fibroblasts (21.67 ± 3.92 kb, $n = 5$). Note that telomeres were elongated in all groups compared to the morula stage and that in contrast to the differences in telomere length at the morula stage, there was no significant difference between the different groups of blastocysts. (*F*) A sequential analysis of telomere length in embryos derived from a single *in vitro* fertilization experiment showed telomere elongation at the morula–blastocyst transition, with an average of 15.8 ± 3.1 kb for morula stages ($n = 6$) and 28.7 ± 7.4 kb for blastocyst stages ($n = 6$). Dotted line in *B–D* indicates mean value.

B, and *E*). Telomeres were shorter in all groups of morulae compared to donor cells or germ cells, indicating that there was no significant selection in favor of cells with longer telomere reserves during the cloning process.

Next, telomere lengths were analyzed in blastocysts derived from *in vitro* fertilization or somatic cloning. In all groups, blastocysts had significantly elongated telomeres relative to those at the morula stage (Fig. 3), indicating that telomeres are elongated at this particular stage of bovine embryogenesis. Similarly, a sequential analysis of telomere length in embryos derived from a single *in vitro* fertilization experiment showed significant telomere elongation in embryos of an identical genetic background at the morula–blastocyst transition (Fig. 3*F*). In accordance with previous studies, we did not detect telomerase activity at the morula stage. In contrast, a strong up-regulation of telomerase was observed at the blastocyst stage (3–5), indicating that telomere elongation correlated with the timing of telomerase reactivation during embryogenesis. Telomere lengths of bovine blastocysts were similar regardless of whether they were derived from somatic cloning or *in vitro* fertilization (Fig. 3). These data provide experimental evidence for an embryo-specific telomere elongation program, which leads to restoration of telomere length in both cloned embryos and embryos produced by *in vivo* or *in vitro* fertilization.

In line with these observations, telomere length analysis on 1- to 2-year-old cloned cattle derived from fetal or adult donor cells revealed a similar telomere length when measured in WBC or ear biopsies compared to those of age-matched control animals (data not shown). Because WBC are derived from the telomerase-positive hematopoietic compartment, these data indicate that telomere elongation in cloned cattle is restricted to a defined stage of embryogenesis and does not result in abnormally long telomeres in telomerase-positive compartments. The telomere length of WBC and ear biopsies was comparable between clones derived from adult or fetal fibroblasts, indicating that the cell source did not significantly affect telomere length in the cloned offspring.

Telomere Elongation During Early Mouse Embryogenesis Is Telomerase-Dependent. To test whether the observed telomere elongation is a general mechanism in mammalian development and whether it

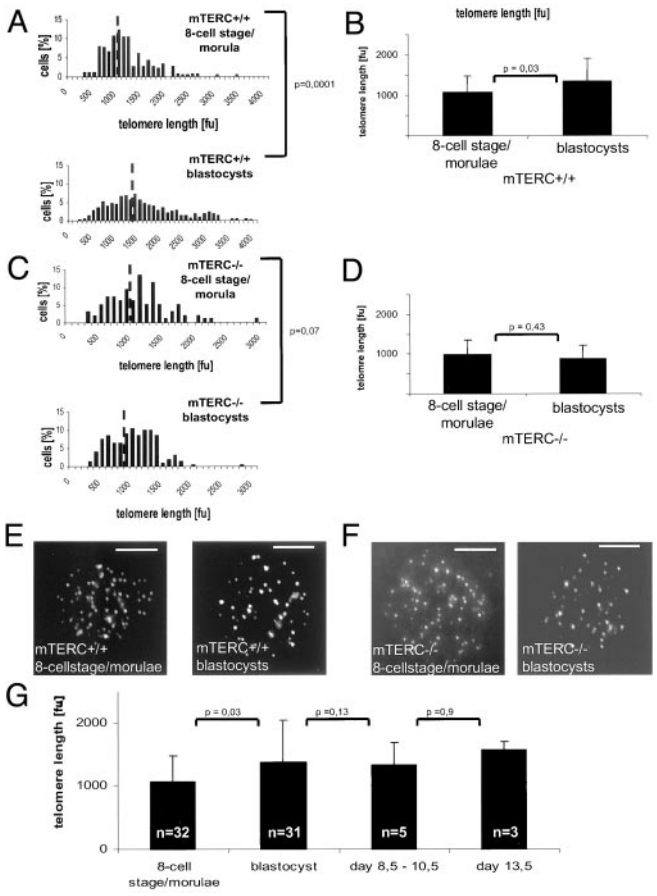

Fig. 4. Telomere length in embryos from mTERC$^{-/-}$ and mTERC$^{+/+}$ mice by Q-FISH. (*A*) The distribution of mean telomere fluorescence units (fu) of all analyzed nuclei from wild-type embryos at the 8-cell/morula stage (1118 ± 484 fu, $n = 274$) and at the blastocyst stage (1501 ± 753 fu, $n = 681$). (*B*) Mean value of the mean telomere fluorescence intensity determined for individual embryos at the 8-cell/morula stage (1,066 ± 406 fu, $n = 32$) and at the blastocyst stage (1,350 ± 565 fu, $n = 31$). (*C*) The distribution of mean telomere fu of all nuclei from telomerase knockout embryos (mTERC$^{-/-}$) at the 8-cell/morula stage (1,060 ± 503 fu, $n = 96$) and at the blastocyst stage (949 ± 388 fu, $n = 199$). (*D*) Mean value of the telomere fluorescence intensity determined for individual embryos at the 8-cell/morula stage (995 ± 356 fu, $n = 14$) and at the blastocyst stage (877 ± 348 fu, $n = 10$). Note that mTERC$^{+/+}$ embryos showed telomere elongation at morula/blastocyst transition, whereas embryos from telomerase-deficient mice (mTERC$^{-/-}$) did not show a lengthening of telomeres but rather a slight decrease in telomere length. (*E* and *F*) Representative photographs of telomere spots in nuclei of mTERC$^{+/+}$ (*E*) and mTERC$^{-/-}$ (*F*) embryo at 8-cell stage/morula and blastocyst stage of embryonic development. Magnification bar, 10 μm. (*G*) Mean value of the telomere fluorescence intensity determined for individual embryos at the 8-cell/morula stage, blastocyst stage, day 8.5/10.5 (1,329 ± 353 fu, $n = 5$), and day 13.5 (1,573 ± 133 fu, $n = 5$). Analysis revealed no significant increase in telomere length at these postimplantation stages ($P > 0.05$). Dotted line in *A* and *C* indicates mean value.

is telomerase-dependent, we analyzed telomere length in early embryogenesis of mTERC$^{-/-}$ and mTERC$^{+/+}$ mice. In agreement with the findings from bovine embryos, the telomeres of mTERC$^{+/+}$ embryos were significantly elongated at the blastocyst stage compared to morulae and 8-cell stages (Fig. 4 *A*, *B*, and *E*). In contrast, in mTERC$^{-/-}$ embryos, telomeres were not elongated during the morula–blastocyst transition (Fig. 4 *C*, *D*, and *F*), indicating that telomerase activity is required for telomere elongation at this stage of embryogenesis. To determine whether telomere elongation is restricted to the morula–blastocyst transition or is a continuous process during early development, we analyzed telomere length of primary cultures of adherent growing cells derived from embryos at days 8.5 (Theiler's stage 13), 10.5 (Theiler's stage 17), and 13.5 of mouse embryogenesis. Analysis revealed no significant increase in telomere length at these postimplantation stages compared with length at the morula–blastocyst transition (Fig. 4*G*), indicating that telomere elongation during embryogenesis is restricted to the morula–blastocyst transition.

Discussion

The discovery of telomere elongation at the morula–blastocyst transition in embryos from two mammalian species as well as embryos derived from either *in vivo* or *in vitro* fertilization and even somatic nuclear transfer indicates a general program in preimplantation development. Morula–blastocyst transition is a critical step in preimplantation development, leading to first differentiation into two cell lineages, the inner cell mass and the trophoblast. This coincides with a dramatic change in morphology from the compacted morula to the cavity-filled blastocyst stage and is associated with massive changes in embryonic gene expression (32). Our study shows that telomeres of cloned embryos derived from fetal or adult fibroblasts are normalized by the telomere elongation program at the morula–blastocyst transition, indicating that this program resets telomere length to a specific set point. Fibroblasts are a predominantly used donor cell type in somatic nuclear transfer in farm animals. It remains to be determined why telomere length in cloned embryos derived from epithelial cell lines is not always restored (27).

Telomeres are not elongated in mTERC$^{-/-}$ mouse embryos, indicating that the telomerase enzyme is required for telomere elongation during early embryogenesis. These data coincide with previous reports on haploinsufficiency of the telomerase RNA component (TERC) in mTERC$^{+/-}$ mice, leading to offspring from intercrosses of mouse strains with different telomere lengths (20, 21). However, because telomerase activation alone does not necessarily result in telomere elongation *in vitro* (34), other factors such as telomere binding proteins could be involved in telomere length regulation at this stage of embryogenesis (35). Interestingly, the telomere elongation program discovered in the present study correlates with activation of telomerase at the same stage of human embryogenesis (4). In bovine-cloned and parthenogenetic embryos, telomerase activity followed the same pattern with a significant up-regulation at the blastocyst stage (36). Telomere elongation during embryogenesis could be critical to ensure normal telomere length segregation from one generation to the next and could have direct effects on regeneration, aging, and carcinogenesis during postnatal life. A detailed understanding of this embryonic telomere-elongation program could ultimately be important for the use of cell transplantation and gene therapy approaches for the treatment of degenerative disorders.

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